Definition of Three Classes of Binding Sites in Isolated Microtubule Crystals†

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ABSTRACT: Isolated vinblastine-induced microtubule crystals have been used to study the stoichiometry of drug and nucleotide binding to microtubule protein. One mole of vinblastine is bound per mole of dimer (mol wt 110,000). Two moles of guanosine nucleotide is bound per mole of dimer. Both the vinblastine and nucleotide are very tightly bound; neither is exchangeable under conditions in which the microtubule crystals are stable. The crystals also bind 1 mole of colchicine/mole of dimer at a third site. The colchicine binding is freely reversible, stereospecific, and temperature sensitive. For the binding reaction in the 0–20° range, ΔH_0 = 16 kcal/mole and ΔS_0 = 79.5 eu. Competition experiments indicate that another antimitotic agent, podophyllotoxin, competes for the colchicine site. Experiments designed to test for possible phosphorylation of the tubule subunits in the crystals or binding of cyclic nucleotides by the crystals were unsuccessful. The stoichiometries of binding are interpreted in terms of a heterodimer model of microtubule subunits, with each chain containing only one drug binding site and one nucleotide site.

Treatment of various cell types with the antimitotic alkaloid vinblastine sulfate results in the induction of highly organized paracrystalline inclusion bodies (Bensch and Malawista, 1969; Malawista *et al.*, 1968; Krishan and Hsu, 1969; Schochet *et al.*, 1968; Wisniewski *et al.*, 1968). Morphological evidence (Bensch and Malawista, 1969) and direct chemical characterization (Bryan, 1971a, 1972) of the isolated crystalline structures indicate that they are composed of equimolar amounts of two closely related polypeptide chains which are indistinguishable from microtubule subunits. Microtubules, in the uncrystallized state, are a common cytoplasmic feature of all eucaryotic cells and are involved in a variety of cellular processes including chromosome movement (Inoue and Sato, 1967), axonal transport (Kreutzberg, 1969), ciliary and flagellar movements (Satir, 1968), and the maintenance and development of cell form (Tilney and Gibbins, 1969; Goldman, 1971). Studies on microtubule subunits, isolated using a different antimitotic drug, colchicine, as a label, indicate that the "native" receptor is a dimeric unit (Weisenberg *et al.*, 1968; Shelanski and Taylor, 1968). Recent work indicates that this receptor is a heterodimer (Bryan and Wilson, 1971; Bryan, 1972; Feit *et al.*, 1971; Fine, 1971). The present report deals with the binding properties of these heterodimers in the intact vinblastine-induced crystals. The crystals contain three distinct types of binding sites, one for vinblastine, one for colchicine (or podophyllotoxin), and one for guanosine nucleotides. In view of the proposal that cAMP mediates phosphorylation (Goodman *et al.*, 1970) of microtubule subunits, attempts were also made to demonstrate binding of cyclic nucleotides or phosphorylation of the intact crystals.

Materials and Methods

The eggs of the sea urchin *Stronglyocentrotus purpuratus* were used. The procedures for obtaining gametes and the methods used to induce and isolate crystals have been described (Bryan, 1971b).

Binding Experiments. The binding experiments were done by incubating one to four nanomoles of crystals in 100–200 μl of the appropriate solution. Incubations were done in Reacti-vials (0.3-ml total volume, Pierce Chemical Co.). After 18–24 hr the contents of the vial were evenly suspended by vortexing and aliquots were removed to determine total ligand concentration. After centrifugation at 3000*g* for 10 min, the supernatant was sampled to determine unbound ligand. The amount of bound ligand was calculated from the difference between total and free-ligand concentrations. Binding experiments were done using [^{3}H]colchicine (170 Ci/mole) (a gift from Dr. Leslie Wilson), [8-^{3}H]adenosine-3′,5′-cyclic phosphate (150 Ci/mole) (Amersham/Searle), [^{3}H]guanosine 3′,5′-cyclic phosphate (230 Ci/mole) (Schwartz/Mann), and [8-^{3}H]guanosine 5′-triphosphate (200–500 Ci/mole) (Amersham/Searle).

Certain experiments, particularly those on the kinetics of colchicine binding were done by incubating 2.0 ml of a crystal suspension with [^{3}H]colchicine. At appropriate time intervals, 0.25-ml aliquots were removed and centrifuged; the resulting crystal pellet was then washed twice to remove excess unbound colchicine before counting.

Preparation for Counting. Samples of crystals (or supernatants) were transferred to scintillation vials, then solubilized by addition of 250 μl of NCS (Amersham-Searle). The solubilized mixture was then suspended in 10 ml of scintillation fluid (5 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 0.3 g of 2,5-diphenyloxazole in 1.0 l. of toluene) and counted.

Determination of Bound Vinblastine and Nucleotides. The amount of vinblastine bound to the crystals was determined by two methods. Crystals were formed *in vivo* by incubation with [^{3}H]vinblastine (a gift from Dr. Leslie Wilson). After isolation and purification, known amounts of intact crystal proteins were transferred to scintillation vials then solubilized and counted. Alternatively, isolated crystals (10–15 mg) were sequentially extracted with 2.0 ml of 80% aqueous 2-

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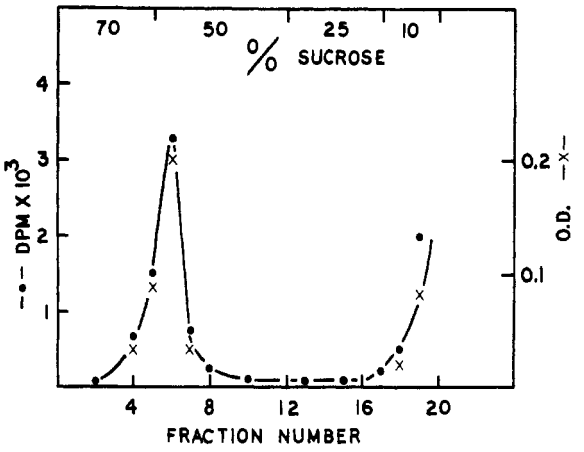

FIGURE 1: Sucrose density step gradient of vinblastine induced crystals prelabeled with [^{3}H]colchicine. One milligram of isolated purified crystals was incubated for 2 hr (13°) in 1.0 ml of 100 mM KCl, 1 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4) containing 1.2 μM [^{3}H]colchicine (170 Ci/mole). The reaction was terminated by layering the incubation mixture on top of a preformed step gradient (70, 50, 25, 10% w/w sucrose in 100 mM KCl, 1 mM $MgCl_2$, and 10 mM Tris-HCl, pH 6.8) and centrifuging in the SW-25 rotor (Spinco) at 24,000 rpm for 4 hr. The run was terminated and fractions (20 × 1.2 ml) collected. Aliquots of each fraction were read at 330 nm or dissolved in NCS and counted.

propanol (25°) and 2.0 ml of 10% perchloric acid (0°). The extracts were pooled and the ultraviolet spectra were measured. The relative amounts of vinblastine (VB) and guanosine nucleotides (GTP) present were evaluated from the optical densities at 260 and 290 nm by solving two simultaneous equations:

$$OD_{290\,nm} = \epsilon_{VB}^{290}[VB] + \epsilon_{GTP}^{290}[GTP]$$

$$OD_{260\,nm} = \epsilon_{VB}^{260}[VB] + \epsilon_{GTP}^{260}[GTP]$$

The millimolar extinction coefficients (ϵ) used were ϵ_{VB}^{290} = 12.1, ϵ_{VB}^{260} = 10.5, ϵ_{GTP}^{290} = 11.4, and ϵ_{GTP}^{260} = 5.8.

Some caution must be used in the extraction steps since vinblastine perchlorate is only marginally soluble having a measured concentration at saturation in 10% perchloric acid of about 8.6×10^{-5} M.

Inhibitors. Several reagents were used in an attempt to inhibit the colchicine binding abilities of the intact crystals including iodoacetic acid, *N*-ethylmaleimide, 2-methoxy-5-nitrotropone, and another antimitotic agent podophyllotoxin (Aldrich Chem. Co.).

Phosphorylation Experiments. Attempts were made to phosphorylate both *in vivo* and *in vitro*. The *in vivo* experiments were done by inducing crystals in the presence of $^{32}PO_4$ (1–5 mCi/ml). After isolation, the crystals were sampled directly then precipitated with 10% perchloric acid containing 0.1% pyrophosphate then resuspended in 1 N NaOH. This washing was repeated twice then the final protein pellet was solubilized with NCS and counted. *In vitro* phosphorylation experiments were done using the procedures of Miyamoto *et al.* (1969). The reaction was terminated by addition of 2 ml of 10% perchloric acid (0°) containing 0.1% pyrophosphate, the precipitate was collected by centrifugation and redissolved in 1 N NaOH. This washing procedure was repeated twice and the final pellet solubilized with NCS and counted.

Protein Determinations. Protein assays were done using the method of Lowry *et al.* (1951). Crystals for protein assay were washed twice with 10% perchloric acid (0°) to remove vinblastine which interferes with the assay. Vacuum-dried, reduced, and acetylated microtubule protein was used as a protein standard.

Results

Bound Guanosine Nucleotide and Bound Vinblastine. The extent of vinblastine binding was determined spectrophotometrically or by using [^{3}H]vinblastine. The extent of nucleotide binding was estimated spectrophotometrically. The [^{3}H]-vinblastine-labeling studies gave 0.98 (±0.05) mole of vinblastine bound per mole of protein. Spectrophotometric determinations gave 0.99 (±0.10) mole of vinblastine bound per mole of protein and 2.05 (±0.15) moles of GTP bound per mole protein. The figures were calculated using a dimer molecular weight of 110,000. The values are the average of four separate experiments for each method. For convenience, and on the basis of previous reports (Weisenberg *et al.*, 1968; Stephens *et al.*, 1967), the guanosine nucleotide was assumed to be GTP. If the vinblastine in the perchloric acid extracts is removed by passage of the extract over AG-50W (Bio-Rad Laboratories), the spectra indicate that the nucleotide is in fact a guanosine derivative with optical density ratios at acid pH of 250/260 = 0.99, 280/260 = 0.70, and 290/260 = 0.50. Adjustment of the extract to neutral or alkaline pH produces the spectral shifts characteristic of guanosine derivatives. A preliminary thin-layer chromatographic characterization of the extract indicates that at least some of the nucleotide is GTP and that the vinblastine is unaltered. Attempts to obtain additional binding of [^{3}H]GTP or [^{3}H]vinblastine to the crystals were unsuccessful. These results indicate that in the *in vivo* produced crystals both vinblastine and nucleotides are present. These ligands are not removed by repeated washes in dilute salt solutions and, under the conditions tested, will not exchange with exogenous GTP or vinblastine.

Bound Cyclic Nucleotides. Several attempts using the equilibrium technique were made to obtain binding of exogenous cyclic nucleotides, both 3′,5′-cAMP and 3′,5′-cGMP. No binding was obtained with or without Ca^{2+}, Mg^{2+}, EGTA, mercaptoethanol, NaF, or theophylline. Concentrations of cyclic nucleotide from 10^{-8} to 10^{-4} M were tested.

Phosphorylation. Several attempts *in vitro* were made to achieve phosphorylation of microtubule subunits in the intact crystals. No phosphorylation was observed using the conditions of Miyamoto *et al.* (1969) with or without cyclic AMP. Induction of the crystals in the presence of $^{32}PO_4$ led to the incorporation of label into the intact crystals. This label was not removed by the standard isolation conditions (multiple washing with dilute salt solutions) but was completely removed by either solubilization of the crystals with guanidine hydrochloride or urea or by extraction with acid and base as outlined in the Method section. This label is presumably in the nucleotide. No significant incorporation was observed into the subunits themselves.

Colchicine Binding. Initial colchicine binding was demonstrated by incubating a crystal suspension with [^{3}H]colchicine then running the incubation mixture on a sucrose step gradient. After centrifugation at 24,000 rpm (SW-25 rotor) for 4 hr, fractions were collected and aliquots were assayed for the presence of crystals (OD 330 nm) or solubilized with NCS and counted. Figure 1 indicates the results of a discontinuous sucrose gradient (10, 25, 50, 70% w/w). The crystals band out on the 70% layer and are visible in the centrifuge tube as an

TABLE I. Effects of Irradiation on the Binding Properties of Vinblastine Induced Crystals.

Experiment	Dpm	% Control
Colchicine bound, no irradiation	3300	100
Colchicine bound, irradiation	520	16
Incubation with lumicolchicine	75	2
Preirradiation, colchicine bound	3200	97

opalescent interface. The colchicine label clearly follows the crystals.

Irradiation. To ensure that this labeling was qualitatively similar to that observed in other systems (Wilson and Friedkin, 1967), experiments were done to demonstrate that certain derivatives of colchicine, the lumicolchicines, would not bind. Two types of experiments were done. Crystals were incubated with [^{3}H]lumicolchicine prepared by exposing [^{3}H]colchicine (4.5×10^{-5} M) to uv irradiation (350 nm) for thirty minutes. Alternatively, crystals were prelabeled with [^{3}H]colchicine, then suspensions were split into equal aliquots, one for irradiation and one for a control. Irradiation was done by placing one aliquot of suspension approximately 1–1.5 mm in depth in a small flat dish and irradiating for 15 min by placing a long-wavelength (350-nm) ultraviolet lamp at 3 cm from the surface. After irradiation (or incubation with irradiated colchicine), the samples were washed twice and the resulting crystal pellets solubilized and counted. The results are given in Table I along with a control experiment showing that previously irradiated crystals maintain binding activity.

Stability. One of the problems associated with previous attempts to determine the number of colchicine binding sites has been the extreme lability of the colchicine receptor (Weisenberg *et al.*, 1968; Wilson, 1970). To ascertain the degree of lability of the colchicine site in the crystal preparations, equilibrium experiments were done as described in the Method section but aliquots were removed at increasing time intervals. The crystals in stabilizing medium alone (100 mM KCl–1 mM $MgCl_2$–10 mM Tris-HCl (pH 7.2)) or stabilizing medium plus 10^{-4} M vinblastine and 1 mM GTP had no observable decay over the 5-day period tested. This should be compared to the *in vitro* half-life of the dimer in brain (about 6–12 hr; Weisenberg *et al.*, 1968) or sea urchin egg homogenates (5–10 hr; L. Wilson, personal communication).

Kinetics. A preliminary examination of the kinetics of binding of colchicine was done in order to determine what incubation times were appropriate to ensure equilibrium. Crystal suspension was incubated with 4.5×10^{-6} M colchicine, aliquots were removed at intervals, and the crystals were washed by centrifugation then solubilized and counted. The results are shown in Figure 2. The "plateau" region is reached in approximately 8–10 hr at 15°. For equilibrium measurements, incubations were routinely allowed to proceed for 24–36 hr.

Equilibrium Measurements. Equilibrium measurements were analyzed in terms of the reaction: protein + nCLC[1] $\rightleftharpoons$ protein CLC_n as previously suggested by Taylor (1965).

[1] CLC = colchicine.

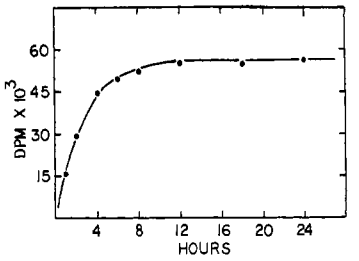

FIGURE 2: Kinetic experiment to determine time course of colchicine binding to intact crystals. Eight milligrams of crystals were incubated (15°) in 2.0 ml of 100 mM KCl, 1 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4) containing 4.5 μM [^{3}H]colchicine (170 Ci/mole) at the appropriate time intervals 0.25-ml samples were withdrawn and washed three times with 50 volumes of the same medium containing no colchicine. The final pellet was then dissolved in NCS and counted. The total "transfer" time was approximately 30 min for each sample; no attempt was made to correct for possible losses during the washing procedures.

The results of several experiments at different colchicine concentrations and several temperatures are shown in the form of Scatchard plots (Scatchard, 1949) in Figure 3. The fractional number of moles of occupied binding sites/mole of protein ($\bar{V}$) was calculated at each colchicine concentration using a molecular weight of 110,000. A composite value for n, the extrapolated number of binding sites per molecule at infinite colchicine concentration, was determined from twelve independent binding experiments at nine different temperatures. This was $n = 1.07$ (0.05) moles of colchicine bound per 100,000 g of protein. The value in parenthesis is the variance at the 95% confidence level calculated using the Students' t test (Laitinen, 1960). This value of n is consistent with one colchicine site per "dimeric" unit in the intact crystals.

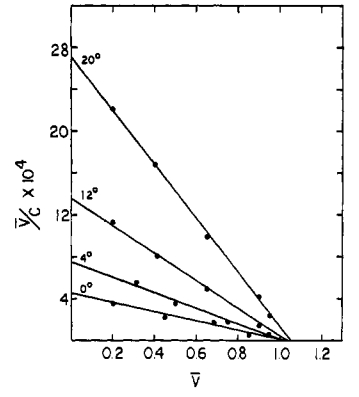

FIGURE 3: Scatchard plots of the binding data at different temperatures. The data shown are representative of a dozen experiments at different temperatures. Approximately three nanomoles of crystals (for each point) were incubated at the indicated temperatures in 200 μl of 100 mM KCl and 1 mM $MgCl_2$ buffered with either 10 mM Tris-HCl (pH 7.4) or 10 mM potassium phosphate (pH 6.8). Incubations were done in Reacti-vials (Pierce Chemical Co., 0.3-ml total volume) using varying concentrations (10^{-6}–10^{-4} M) of [^{3}H]colchicine (170 Ci/mole). The reactions were terminated between 24 and 36 hr. Free and total [^{3}H]colchicine concentrations were determined as described in the Methods section. Bound [^{3}H]colchicine was calculated from the difference between total and free ligand. $\bar{V}$ is the fractional number of occupied sites per protein molecule, calculated using a molecular weight of 110,000. [C] is the concentration of free colchicine. The extrapolated value of $\bar{V}$ for twelve experiments was $n = 1.07$ (0.05). The parenthesis indicate the variance at the 95% confidence level as calculated using the Students' t test.

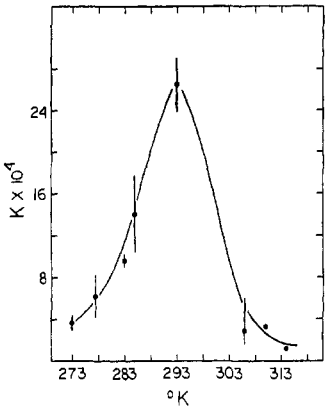

FIGURE 4: Temperature profile f[illegible] the equilibrium constants of the binding reaction. The association constants (K) are taken from the extrapolated values of $\bar{V}/[C]$ from Figure 4 using Scatchard's form of the mass action law: $\bar{V}/[C] = k(n - \bar{V})$ and assuming $n = 1.00$. The error bars represent the range of values for extrapolations at each temperature. The temperature maximum is approximately 20° (293°K) for the conditions used.

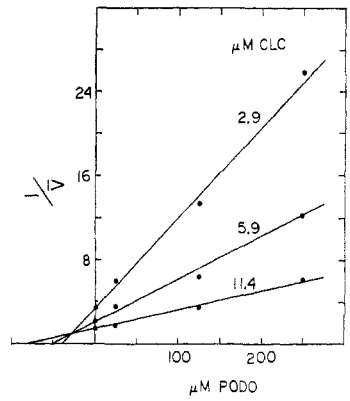

FIGURE 5: Modified Dixon plot demonstrating the effect of podophyllotoxin on the colchicine binding reaction. The concentration of podophyllotoxin present is plotted *vs.* $1/\bar{V}$ at several initial concentrations of colchicine. The reaction conditions were essentially the same as those described in Figure 4. Two nanomoles of crystals (for each point) was incubated in Reacti-vials in 100 μl of 100 mM KCl, 1 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4) containing the indicated initial amounts of [^{3}H]colchicine (170 Ci/mole)) and podophyllotoxin. After 24-hr incubation at 13°, the reactions were terminated and the amounts of colchicine bound determined as described in the Methods section. The interception of the lines at one point indicates the inhibition is competitive and gives a dissociation constant (K_i) for podophyllotoxin of 30 μM.

The equilibrium constants, k = (protein–CLC)/(protein) × (CLC), determined from the Scatchard plots are given as a function of temperature in Figure 4. The equilibrium constants go through an apparent maximum in the region of 20°, although this maximum was not precisely determined and may occur at a slightly higher temperature. No attempt has been made to see if the decrease at higher temperatures is, in fact, irreversible.

The thermodynamic parameters of the binding reaction in the region of 0–20° were determined by replotting the data in Figure 4 in the form log K *vs.* $1/T$. The values obtained were $\Delta H_0 = 16$ kcal/mole, $\Delta S_0 = 79.5$ eu. At the ambient temperature of the organism (13°) $\Delta F = -6.7$ kcal/mole.

Inhibitor Studies. Several attempts were made to inhibit colchicine binding using common protein modifying reagents. *N*-Ethylmaleimide, iodoacetic acid, and 2-methoxy-5-nitrotropone had no effect on colchicine binding even at millimolar concentrations.

The antimitotic agent, podophyllotoxin, has been reported to reversibly affect the colchicine site in chick embryo brain homogenates (Wilson, 1970). To ascertain if this was a competitive or a noncompetitive effect, colchicine binding experiments were done in the presence of increasing amounts of podophyllotoxin. The amounts of colchicine bound were determined using the equilibrium technique. The results are given in Figure 5 as a modified Dixon plot; $1/\bar{V}$ *vs.* podophyllotoxin concentration. The observed behavior is that expected for a competitive inhibitor (Dixon, 1953), and is consistent with the notion that podophyllotoxin and colchicine compete for the site on the dimer. The results further indicate that the dissociation constant for podophyllotoxin is approximately 30 mM. This indicates that the receptor site has approximately one-quarter the affinity for podophyllotoxin as it has for colchicine (K(PODO)/K(CLC) = 33/141 = 0.23) at 13°

Discussion

The present results demonstrate that the vinblastine-induced crystals have three distinct classes of binding sites. One class of sites is occupied by guanosine nucleotides, a second is occupied by the dimeric alkaloid vinblastine, while the third class of sites recognizes colchicine, podophyllotoxin, and presumably colcemid. The results confirm previous reports on the stoichiometries of nucleotide and colchicine binding and demonstrate that one molecule of vinblastine is also bound per dimer. These three classes of sites are independent in the sense that no competition is observed between the ligands for the same site.

Nucleotide Sites. Several workers have reported that microtubule subunits contain guanosine nucleotides. Stephens *et al.* (1967) have reported the presence of varying amounts of GTP, GDP, and GMP in sea urchin flagellar outer fibers, while Weisenberg *et al.* (1968) have reported that purified colchicine receptor from adult mammalian brain has two nucleotide sites per dimeric unit of 120,000 molecular weight. These two sites did not appear to be equivalent since one nucleotide was freely exchangeable with exogenous nucleotide while the other was nonexchangeable under the conditions used. Berry *et al.* (1971) have further indicated that the nonexchangeable nucleotide may dephosphorylate upon polymerization. The exchangeable site was labile in the brain preparations with a half-life of approximately 11 hr. Although the present experiments were done using intact crystals, the calculated nucleotide stoichiometry is consistent with 2 sites per dimer of 110,000 molecular weight. Extrapolating from the results of Weisenberg *et al.* (1968), both of the binding sites on the dimer are apparently occupied in the crystals. Interaction of the dimer with vinblastine or its insertion into structure has reduced or eliminated the lability of the second site and eliminated reversible exchange with exogenous nucleotide.

Vinblastine Site. The effects of vinblastine on intact cells, cell homogenates, purified colchicine receptors, and several types of "structural" proteins have been described. In general, in living cells treatment with vinblastine induces the formation of aggregates of various types. The formation of aggregates of microtubules, filaments, and polyribosomes (Bensch and Malawista, 1969; Malawista *et al.*, 1968; Krishan and Hsu, 1969; Schochet *et al.*, 1968; Wisniewski *et al.*, 1968) have been reported in eucaryotic cells while aggregates of polyribo-

somes have been observed in procaryotes (Kingsbury and Voelz, 1969). *In vitro*, vinblastine affects the precipitation of several types of proteins, particularly Ca^{2+}-precipitable proteins (Wilson *et al.*, 1970; Kleinig *et al.*, 1971) including microtubule proteins (Wilson *et al.*, 1970; Bensch *et al.*, 1969; Marantz *et al.*, 1969; Olmsted *et al.*, 1970; Marantz and Shelanski, 1970). The initial stages of the aggregation of purified microtubule protein have been studied in the ultracentrifuge by Weisenberg and Timasheff (1970). These authors report an enhancement of aggregation by Mg^{2+} and finding a variable number of vinblastine molecules bound per dimer, but never more than two. As previously indicated, crystals produced *in vivo* appear to have only a single bound vinblastine molecule per microtubule dimer.

Colchicine Site. Previous work has demonstrated that the colchicine receptor is a subunit of microtubules. In addition, the binding reaction itself is known to be stereospecific, temperature sensitive, and influenced by several other antimitotic drugs. The colchicine binding reaction of the vinblastine induced crystals exhibits these same characteristics. The advantage of using this "crystalline" form of microtubule subunits to investigate colchicine binding lies in the apparent stability of the binding site in the aggregates. It is not yet clear if the increased stability is due to a conformational change induced by vinblastine within the dimer or if it is a function of dimer aggregation *per se*.

The kinetics of colchicine binding are slow, usually requiring several hours to go to completion at micromolar concentrations. This does not appear, however, to be a restricted diffusion within the crystals as similar data have been obtained in free solution (Wilson and Friedkin, 1967; Borisy and Taylor, 1967b) in different organisms in the absence of vinblastine. This may indicate that colchicine binds to a conformation of the dimer which is energetically unfavorable.

Several values for equilibrium constants for colchicine binding in free solution are available (Borisy and Taylor, 1967a,b). A direct comparison is somewhat difficult, however, since different organisms and techniques were used. For another species of urchin (*Arbacia punctulata*), Borisy and Taylor (1967b) obtained a value of $K = 2.3 \times 10^6$ l. mole^{-1} at 37° This is approximately 100 times greater than the K values found in the crystals from *S. purpuratus* at 37°, and ten times greater than the maximum K at 20°. It is not yet clear if the binding of vinblastine to the dimer reduces the dimer's affinity for colchicine, or if the *A. punctulata* values are elevated as a result of the difficulties involved in working with labile binding sites.

The colchicine binding reaction itself is temperature dependent with positive enthalpy and positive entropy changes and a relatively large favorable free energy change. These parameters are similar to those measured for the binding of other small apolar molecules and are consistent with the notion that colchicine is perhaps bound in a hydrophobic or nonpolar pocket.

Interactions between Sites. Two types of interactions between the sites are quite clear. First, vinblastine apparently acts on one nucleotide site to reduce or eliminate the "exchangeability" of the site and secondly, vinblastine acts on both the nucleotide and colchicine sites to block the decay reactions which Wilson (1970) has indicated are irreversible and probably due to denaturation of the subunit(s). The nucleotide and colchicine sites also interact; excess exogenous GTP increases the half-life of the colchicine site while bound colchicine increases the half-life of the nucleotide site (Weisenberg *et al.*, 1968). Since it is somewhat difficult to visualize how rapid subunit decay could be tolerated *in vivo*, one interpretation of the interactions and binding data is that the vinblastine site is a regulatory site normally occupied by a small effector molecule which controls polymerization.

Cyclic Nucleotides and Phosphorylation. A consideration of current work (Goodman *et al.*, 1970) indicating that microtubule subunits can be phosphorylated *in vitro* by a cAMP-dependent mechanism, and the suggestion that the subunits themselves might have protein kinase activity argued that the crystals might possess either kinase activity or be capable of interacting with cyclic nucleotides. The results, however, indicate that the intact crystals do not appear to bind cyclic nucleotides under the conditions tested nor could the crystallized subunits be induced to phosphorylate. Unfortunately, the possibility that vinblastine may inhibit any presumptive phosphorylation has not been ruled out, so it is difficult to argue that the subunits in fact have no intrinsic kinase activity.

Correlation with Protein Structure. Recent work (Bryan and Wilson, 1971; Feit *et al.*, 1971; Fine, 1971; Olmsted *et al.*, 1971) on the biochemistry of microtubule subunits from several sources, including vinblastine induced crystals (Bryan, 1972), indicates that the subunits are heterogenous and that the colchicine binding dimer is probably composed of nonidentical monomers. This notion and the stoichiometry of ligand binding suggests that a soluble heterodimer will have four sites, two of which bind guanosine nucleotides, one which binds vinblastine and one which will bind colchicine or podophyllotoxin. How are these three classes of sites distributed between the two chains? Although interactions between the different classes of sites have been demonstrated, no information is available concerning which chains contain which binding sites. The simplest model to account for the available data would be a heterodimer; one monomer which can bind a nucleotide and vinblastine, while the other monomer binds colchicine (or podophyllotoxin) and another nucleotide. The model would predict that affinity labels of colchicine or vinblastine should only mark a single chain, and that the sequence differences between the two chains may be confined to the regions around the drug binding sites. This is in agreement with the close similarity of amino acid compositions (Bryan and Wilson, 1971; Bryan, 1972) and peptide maps (Fine, 1971).

Acknowledgments

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Proteins Exposed on the Surface of Mammalian Membranes†

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ABSTRACT: The technique employing lactoperoxidase-catalyzed iodination for investigating protein distribution within erythrocyte stroma has been extended to examine the proteins of the surface membranes of mouse fibroblasts (L cells). The surface membranes were examined after four different conditions of iodination: (1) the intact cell, where only proteins exposed at the surface are susceptible to iodination by lactoperoxidase; (2) the intact cell in the absence of lactoperoxidase as a measure of nonspecific iodination; (3) the isolated surface membrane; and (4) the partially denatured surface membrane, where, presumably, most proteins are accessible. The surface membranes were solubilized in sodium dodecyl sulfate and the protein subunits were examined by disc gel electrophoresis. The results indicate that one group of polypeptides with a molecular weight of approximately 230,000 is in an exposed position on the exterior of the L cell surface membrane. Smaller amounts of radioactive iodine are associated with polypeptides of molecular weight 46,000–150,000 suggesting that a small portion of their chain is exposed. No radioactivity is associated with two polypeptides of large molecular weight (170,000 and 190,000), a polypeptide of molecular weight 39,500, and several smaller polypeptides of molecular weight range 15,000–32,000. All proteins are iodinated when surface membranes were partially denatured and subsequently iodinated. The fact that the degree of iodination increases manyfold in the isolated membranes supports the observation that only a few proteins are exposed on the surface of the intact L cell. This iodination technique was extended to the surface membranes of baby hamster kidney fibroblasts, BHK_{21}/C_{13}, before and after transformation by the Bryan strain of Rous sarcoma virus, C_{13}/B_4. Again the results indicate that in both cases a large molecular weight group of polypeptides is exposed on the outside of the surface membrane.

The paucity of information regarding the spatial arrangement of proteins in membranes can be attributed to inadequate techniques of protein vectorial analysis. The recent report of Phillips and Morrison (1971a) regarding the use of lactoperoxidase to iodinate membrane proteins by means of an enzyme–substrate complex which is impermeable to erythrocyte membranes (Phillips and Morrison, 1970) seemingly circumvents this problem. This technique has been extended

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